OXIDATION IN HEALTHY AND DISEASED APPLE BARK

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During an investigation of oxidase activity in the bark of trees affected with Illinois canker, caused by *Nummularia discreta* (Schw.) Tul., there appeared an interesting correlation between oxidation, as measured by BUNZEL'S simplified apparatus (1), and the acidity of the bark extracts. These were made, in a manner to be described later, from material of three different kinds: (1) green bark from limbs of trees unaffected by canker; (2) green and still seemingly healthy bark from trees badly affected by canker; (3) brown diseased bark from around the edges of cankered areas.

Bark obtained during the winter months did not separate easily from the wood, but care was taken to make the removal as complete as possible without scraping or cutting off any of the wood. While the diseased bark may have contained fungus parasites other than *Nummularia*, the precautions taken make this unlikely. All of the really dead dry bark on the surface of the canker was cut and scraped away, until the moist brown or blackened bark around the edge was reached. A strip of this 3–4 cm. wide, including sometimes a little of the green bark next to it, was then cut off down to the wood and used as "diseased bark." The various samples obtained were dried to constant weight at 65–70° C., ground up almost to a dust with a meat-grinder, and stored in glass-stoppered museum jars or tightly corked flasks. The writer realizes, of course, the need of tests on extracts from undried bark, and expects to carry through a series of them as soon as time and other work will permit.

Extracts for the various experiments were prepared under conditions which made them quantitatively comparable. In all cases distilled water was added at the rate of 8.5 cc. per gram of dried ground bark, and toluene at the rate of 0.5 cc. per 100 cc. of water used. The beaker containing the mixture was set on top of an incubator at a temperature of about 29° C. for one hour, during which time the mixture was thoroughly stirred five or six times.

The extract was then filtered through four thicknesses of cheesecloth, and finally through S. & S. filter paper no. 588.

As previously stated, all tests were conducted with BUNZEL'S simplified oxidase apparatus. In this there is no provision made for absorption of the carbon dioxide produced, hence only comparative results can be obtained. It is probable, too, as BUNZEL points out (**2**, p. 30), that with no separate alkali solution in the apparatus a longer time is required for the reaction to come to an end, because of the slower absorption of the carbon dioxide by the mixture in the apparatus. This may partly account for the time required in the experiments described later, but it seems unlikely that a mercury rise which continued for 21 days in the absence of a means for absorbing carbon dioxide would have ceased within a few hours with such a means present. So far as BUNZEL'S (**2**) published data are concerned, there is no evidence that oxidation would not have continued longer if his experiments had covered a longer period. REED (**3**) and APPLEMAN (**4**), using BUNZEL'S larger apparatus, though they do not state whether or not they used the alkali basket, set the limit for completion of the reaction at 2–4 days.

Corrections for temperature variations were made by running with each experiment a blank apparatus containing only water, and subtracting from the readings in the others the reading above zero (negative pressure) in the blank. No readings were taken when the mercury in the blank stood below zero (positive pressure). It was feared that discrepancies might be introduced by the temperature variations, but as a matter of fact the differences between the results in any two comparable experiments were found to be no larger than those between duplicate apparatus in the same experiment.

As a preliminary experiment (no. 1) an extract was prepared, as already described, from dried ground bark (sample 17) of a healthy Ben Davis limb 3 inches in diameter, and the apparatus set up as follows: 8, 9, 10, 11, 12, with 1 cc. ext.+1 cc. H_2O+4 cc. 1 per cent pyrogallol; 13, with 6 cc. of distilled water.

The shaking was performed by tipping back and forth six or eight times the wire culture-tube holder in which the oxidase appara-

tus were fastened. Oxidation, as indicated by rise of mercury (negative pressure really), was not complete even at the end of seven days, as was well demonstrated by later experiments.

TABLE I

MANOMETER READINGS IN EXPERIMENT NO. 1; EXTRACT OF HEALTHY BARK, SAMPLE 17

DAY OF TEST	TIME OF READING	ELAPSED TIME IN HOURS AND MINUTES	TEMPERATURE AT TIME OF READING	MANOMETER READINGS CORRECTED AGAINST AN APPARATUS CONTAINING ONLY WATER					
				Extract unchanged					H_2O
				8	9	11	12	10	13
1st...	3:55 P.M.	0*	36.0	0.00	0.00	0.00	0.00	0.00	0.0
2d...	9:30 A.M.	17:35	35.5	−0.15	−0.20	−0.25	−0.20	−0.20	0.0
	2:30 P.M.	22:35	36.0	−0.20	−0.25	−0.35	−0.30	−0.20	0.0
3d...	5:00 P.M.	49:05	35.0	−0.60	−0.60	−0.66	−0.60	−0.60	0.0
4th...	2:37 P.M.	70:42	33.9	−0.80	−0.75	−0.93	−0.82	−0.90	0.0
	5:00 P.M.	97:05	34.0	−0.80	−0.78	−0.92	−0.95	−0.98	0.0
5th...	8:50 A.M.	112:55	32.0	−0.95	−0.90	−1.08	−1.04	−1.08	0.0
6th...	11:25 A.M.	139:30	34.0	−1.25	−1.25	−1.25	−1.15	−1.30	0.0
7th...	9:00 A.M.	161:05	33.0	−1.40	−1.35	−1.35	−1.30	−1.40	0.0

* Put in incubator at 2:55 P.M.; closed and shaken at 3:55 P.M. to mix thoroughly the extract and pyrogallol; shaken again next morning.

A similar experiment (no. 2) was set up, using the extract of diseased bark from another Ben Davis tree in the same orchard. The results are given in table II.

TABLE II

MANOMETER READINGS OBTAINED FROM EXPERIMENT NO. 2; EXTRACT OF DISEASED BARK, SAMPLE 15

DAY OF TEST	TIME OF READING	ELAPSED TIME IN HOURS AND MINUTES	TEMPERATURE AT TIME OF READING	MANOMETER READINGS CORRECTED AGAINST A TUBE CONTAINING ONLY WATER					
				Extract unchanged					H_2O
				8	9	10	11	12	13
1st ..	4:10 P.M.	0*	36.3	0.00	0.00	0.00	0.00	0.00	0.0
2d...	4:12 P.M.	24:02	35.4	−0.99	−0.95	−1.09	−0.85	−0.85	0.0
	4:42 P.M.	24:32	35.6	−1.00	−0.93	−1.10	−0.95	−0.95	0.0
3d...	2:22 P.M.	46:12	36.4	−1.70	−1.45	−1.70	−1.52	−1.50	0.0
	5:09 P.M.	48:59	37.0	−1.75	−1.50	−1.77	−1.55	−1.55	0.0
4th...	4:45 P.M.	72:35	36.0	−1.75	−1.65	−1.75	−1.80	−1.75	0.0
5th...	8:18 A.M.	88:08	36.2	−1.93	−1.75	−1.93	−1.95	−1.90	0.0

* Put in incubator at 3:00 P.M.; all apparatus closed and shaken at 4:10 P.M.; shaken again next morning.

It is seen from table II that oxidation by the extract of diseased bark is more rapid than by the extract of healthy bark, being greater at the end of five days than that caused by healthy extract at the end of seven days.

These two extracts were titrated immediately after extraction with n/20 NaOH, using phenolphthalein as an indicator. The difficulty of determining the end point, due to darkening of the extract on addition of alkali, was avoided by using the test-plate method described by Miss SCHLEY (**5**). In this two phenolphthalein solutions are used, one slightly alkaline, the other slightly acid; the end point is reached when a drop of the solution being titrated just fails to decolorize a drop of the alkaline solution and just shows a faint pink tinge in a drop of the acid solution. By this method the following results were obtained, using 20 cc. of extract in 50 cc. of distilled water: to neutralize 1 cc. of extract of healthy bark to phenolphthalein requires 0.86 cc. n/20 NaOH; to neutralize 1 cc. of extract of diseased bark to phenolphthalein requires 0.38 cc. n/20 NaOH.

These titrations, of course, measure only the base-absorbing power of the extracts, not the H^+ concentration, for the extracts are probably mixtures of strong and weak acids and acid salts, all having different degrees of ionization. But since the concentration of free H^+ ions is known to have a marked influence on many of the reactions taking place in living matter (HÖBER, p. 176), it was important to determine just what this concentration is in the bark extracts. In the absence of facilities for doing this accurately by the gas-chain method, tests were made of the effect of diluted and undiluted extracts on the color changes of various indicators. The most clear-cut results were obtained with mauvein, which is known to give the following color changes: yellow at $H^+ = 2 \times 10^{-3}$; green at 10^{-3}; green-blue at 10^{-4}; blue at 10^{-5}; violet at 10^{-6}. In repeated tests on extracts from different samples of diseased bark, mauvein was turned to a definite green, indicating that here H^+ is about 10^{-3}. Extract of healthy bark turned mauvein yellow, indicating that $H^+ = 2 \times 10^{-3}$. It was found, however, that any given quantity of this extract had to be diluted to 2.4 times its original volume to give the same green as the extract of diseased bark; consequently

(not allowing for increased ionization with dilution), H^+ concentration here would figure, not 2.0 times, but 2.4 times 10^{-3}, or $10^{-2.62}$; that is, both indicator and titration figures show diseased bark to be less acid than healthy bark. With this in mind, a reference to tables I and II above shows that oxidation in the BUNZEL apparatus is in inverse ratio to the acidity of the solution being tested.

This correlation between oxidation and acidity seems also to have obtained in REED'S work (**3**, pp. 56 and 76), though he fails to bring out the point, where juice of bitter rot apples caused greater oxidation than the juice of more acid healthy apples. The results obtained by BUNZEL (**2**, p. 26) in testing the effect of different concentrations of pyrogallol show that as the concentration decreased from 16 per cent to 1 per cent pyrogallol the negative pressure increased from 2.11 cm. of mercury to 2.86 cm. This may not be due to decrease in acidity, as determined by the pyrogallol concentration (attention is not called to it in BUNZEL'S discussion of the table), but such an explanation seems the simplest and most likely.

Titration and indicator tests on a 1 per cent solution of pyrogallol and on a two-thirds of 1 per cent solution, the strength used in the apparatus if the diluent were only water, gave the results shown in table III.

TABLE III

RESULTS OF TITRATION AND INDICATOR TESTS WITH PYROGALLOL SOLUTIONS

PYROGALLOL SOLUTION	NO. OF CC. OF N/20 NaOH NECESSARY TO NEUTRALIZE 1 CC. OF SOLUTION TO PHENOLPHTHALEIN		H^+ CONCENTRATION AS INDICATED BY METHYL ORANGE	
	1 per cent	⅔ of 1 per cent	1 per cent	⅔ of 1 per cent
Fresh	0.13	0.086	$<10^{-3.33}$	$<10^{-3.33}$
6 months old	0.34	0.23	$3.50\times10^{-3.33}=10^{-2.79}$	$2.33\times10^{-3.33}=10^{-2.96}$

The indicator figures for H^+ concentration of a fresh two-thirds of 1 per cent pyrogallol solution (about 1/20 molecular) agree well with those calculated from the electrical conductivity of such a solution. This is known to be 3.12 μ, whence the percentage dissociation $=3.12\div355$ (μ at infinite dilution) $=0.88$ per cent. If

all the dissociation in pyrogallol at 1/20 molecular concentration is due to H^+ dissociation and only one H dissociates, the H^+ concentration is less than 0.000616 equivalent. If all three H's dissociate it is less than 0.000616×3, or, in other words, the theoretical H^+ concentration of the pyrogallol solution in the apparatus is between 10^{-2} and 10^{-3} or less than either. Because of BUNZEL'S statement (**2**, p. 28) that either fresh or old pyrogallol solution may be used it is worthy of note that a 1 per cent solution six months old required dilution to 3.5 and a two-thirds of 1 per cent solution to 2.33 times its original volume before it failed to give the red color with methyl orange, thus indicating for the two-thirds solution an H^+ concentration of $2.33 \times 10^{-3.33}$ or $10^{-2.96}$ (not allowing for increased dissociation with dilution). That is, by slow spontaneous oxidation the H^+ concentration of a two-thirds of 1 per cent solution of pyrogallol solution is increased in six months from less than $10^{-3.33}$ to $10^{-2.96}$. It follows that fresh pyrogallol solution should be made up for each experiment. In this connection it seems rather more than a coincidence that in BUNZEL'S experiment 6 (**2**, table VI, p. 28) fresh 1.0 per cent and 0.1 per cent pyrogallol solutions gave, in a test only one hour long, respectively 0.1 and 0.05 cm. higher negative pressure (greater oxidation) than similar solutions one year old.

The reaction to phenolphthalein of the mixtures in the apparatus may be calculated from the data given in table III. For those containing extract of healthy bark, sample 17, it would be $\frac{0.0+0.86+(4\times 0.34)}{6}=0.37$; for those containing extract of diseased bark, sample 15, $\frac{0.0+0.38+(4\times 0.34)}{6}=0.29$. The acidity of the once distilled water was found to be negligible. On account of the correspondence already observed between titration and indicator figures for both the pyrogallol solution and the extracts, the reaction of the mixtures in the apparatus may be taken to represent roughly the H^+ concentration also until further work can be done.

To test the effect of varying the acidity of the extract on the rate and total amount of oxidation, experiment 3 was set up, using

extract of healthy Ben Davis bark, sample 17. The apparatus contained the following solutions: 8, 9, 10, with 1 cc. extract (acidity reduced one-fourth)+1 cc. H_2O+4 cc. 1 per cent pyrogallol; 13, with 6 cc. distilled water; 11, 12, with 1 cc. extract (acidity reduced one-half)+1 cc. H_2O+4 cc. 1 per cent pyrogallol. The results are given in table IV.

TABLE IV

MANOMETER READINGS IN EXPERIMENT NO. 3; EXTRACT OF HEALTHY BARK, SAMPLE 17

DAY OF TEST	TIME OF READING	ELAPSED TIME IN HOURS AND MINUTES	TEMPERATURE AT TIME OF READING	MANOMETER READINGS CORRECTED AGAINST AN APPARATUS CONTAINING ONLY WATER					
				Three-fourths acid			One-half acid		H_2O
				8	9	10	11	12	13
1st . .	4:40 P.M.	0*	33.0	0.00	0.00	0.00	0.00	0.00	0.0
2d. . .	9:20 A.M.	16:40	33.0	−1.16	−1.14	−0.89	−1.34	−1.32	0.0
	10:00 A.M.	17:20	33.0	−1.19	−1.17	−1.04	−1.55	−1.49	0.0
	4:55 P.M.	24:15	33.5	−1.31	−1.19	−1.14	−1.69	−1.74	0.0
4th. . .	3:50 P.M.	71:10	34.0	−2.05	−1.82	−1.96	−2.59	−2.64	0.0
5th. . .	1:40 P.M.	93:00	32.0	−2.39	−2.27	−2.39	−2.92	−2.92	0.0
6th. . .	4:35 P.M.	119:55	32.0	−2.51	−2.39	−2.59	−3.33	−3.33	0.0
7th. . .	4:20 P.M.	143:40	33.8	−2.71	−2.69	−2.84	−3.44	−3.42	0.0
8th. . .	5:50 P.M.	169:10	34.5	−2.94	−2.84	−2.99	−3.49	−3.59	0.0
9th. . .	8:12 A.M.	183:32	33.1	−2.94	−2.84	−3.09	−3.74	−3.79	0.0

*Put in incubator at 3:10 P.M.; apparatus closed and shaken at 4:40 P.M.; shaken again the next morning.

Experiment 4 was arranged with extract of diseased Ben Davis bark, sample 15, as follows: 12, with 1 cc. extract unchanged (acidity 0.38)+1 cc. H_2O+4 cc. 1 per cent pyrogallol; 11, with 6 cc. distilled water; 8, 9, with 1 cc. extract (acidity 0.29)+1 cc. H_2O+4 cc. 1 per cent pyrogallol. It was thought desirable to run the reactions thus close together (see table V) to test the effect of very small differences in acidity on the rate and total amount of oxidation. The results are given in table V, which summarizes the results from all the experiments described above and from others duplicating them in various ways but not described in detail in this paper.

A study of table V shows that when results are averaged from several different experiments the statement made earlier still holds, that unchanged extract of diseased bark causes greater and more

rapid oxidation of pyrogallol than unchanged extract of healthy bark. Moreover, when the acidity of healthy bark extract is reduced the oxidation is increased, but greatly out of proportion to the reduction in acidity.

TABLE V

SUMMARY OF EXPERIMENTS WITH EXTRACTS OF HEALTHY AND DISEASED APPLE BARK, SHOWING EFFECT OF VARIOUS DEGREES OF ACIDITY OF EXTRACT UPON OXIDATION OF PYROGALLOL

DAY OF TEST	Extract healthy bark, sample 17				Extract diseased bark, sample 15			Extract healthy bark, no. 11	Extract diseased bark, no. 22a
	1	2	3	4	5	6	7	8	9
	*(3.66)	(0.86)	(0.65)	(0.43)	(0.44)	(0.38)	(0.32)	(0.73)	(0.34)
	† 0.83	0.37	0.33	0.29	0.30	0.29	0.28	0.35	0.28
1st.	0.00	−0.26	−1.24	−1.72	−0.87	−0.98	−0.95	−0.26	−0.57
2d.	0.00	−0.61	. . . . .	. . . . .	−1.35	−1.60	−1.45	−0.53	−0.94
3d.	0.00	−0.84	−2.04	−2.68	−1.53	−1.73	−1.76	−0.77	−1.20
4th.	−0.05	−1.01	−2.34	−2.92	−1.63	−1.89	−1.85	−0.86	−1.36
5th.	−0.10	−1.25	−2.49	−3.29	−1.88	−1.95	−2.00	−1.07	−1.50
6th.	−0.13	−1.38	−2.77	−3.43	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
7th.	−0.17	. . . . .	−2.90	−3.59	−1.89	−2.13	−2.19	. . . . .	. . . . .
8th.	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
9th.	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	−1.51	−1.94
	1 test	Average of 6	Average of 3	Average of 3	Average of 2	Average of 6	Average of 2	Average of 2	Average of 2

* The figures in parenthesis indicate reaction of extract.

† The figures in this horizontal line indicate reaction of mixture in oxidase apparatus.

Further proof of the sensitiveness of the oxidase to changes in acidity is found in columns 5 and 6 (table V), where a difference of 0.01 cc. of n/20 NaOH gave throughout the experiment a consistently greater oxidation by the less acid mixture. The figures in column 7 seem unreliable and suggest an error in determination of the acidity. Column 1 shows the great reduction in oxidation produced by increasing the acidity of the mixture in the oxidase apparatus to practically that of the unchanged extract. The figures in columns 8 and 9 are from an experiment in which extracts of healthy and diseased bark were tested at the same time; they show again the correlation between acidity and oxidase activity. This experiment was continued for 19 days, although during the last 4 all oxidation, measured by changes in mercury level, had ceased.

On the nineteenth day the negative pressure was released, all the apparatus closed again, and the test continued for three days more. The results are shown in table VI.

TABLE VI

		Healthy	Diseased
Before releasing.....	19th day of test	−2.03	−2.46
After releasing.....	19th " " "	0.00	0.00
" "	20th " " "	−0.12	−0.08
" "	21st " " "	−0.15	−0.11
		Average of 2	Average of 2

Table VI indicates that oxygen supply is a limiting factor as well as acidity. Its importance should be investigated further. On the twenty-first day, when the experiment was finally closed, it was found that the mixture from the two apparatuses containing healthy bark extract and from the other two containing diseased bark extract had increased from an original acidity of 0.38 in the first case, and 0.28 in the second case, to 0.50 in both (acidity expressed here as before in terms of cc. of n/20 NaOH necessary to neutralize to phenolphthalein). Similar results were obtained in two other experiments in which the reaction had come to an end.

It thus appears that the gradual slowing down of the rate of oxidation in these experiments and in others conducted with similar apparatus is due to increasing acidity and not to chemical combination of the oxidase with some substance in solution, as suggested by BUNZEL (**2**, p. 39). If this be true, the evidence is strong that oxidases are true catalytic agents, prevented usually from bringing about indefinite catalysis by the presence or absence of something which acts as an inhibitor. It is probable that just as enzyme hydrolysis of carbohydrates and other substances reaches a condition of equilibrium because of accumulation of the products of hydrolysis, in exactly the same way, in the BUNZEL apparatus at least, oxidation ceases because of inhibition by accumulated oxidation products. These will include carbon dioxide and acetic and oxalic acids if the decomposition of pyrogallol by oxidase is like that induced by alkalies and the salts of heavy metals. In the presence of O_2 alkalies cause pyrogallol to turn brown with the

formation of carbon dioxide and acetic acid; salts of mercury, silver, and gold oxidize pyrogallol to acetic and oxalic acids. It might appear that oxidation in the larger BUNZEL apparatus, when the alkali basket is used, is greater because of the removal of carbon dioxide, which would tend to increase the acidity if allowed to remain. This can hardly be true, however, since carbonic acid has a low dissociation constant and at the temperature of these experiments would be present in the solutions in comparatively low concentration. Its effect on acidity is but slight at best, and the removal of it merely shortens the time required for the reaction to come to an end. Acetic and oxalic acids are both more important, for two reasons: all of the acid formed remains in solution, and if it is acetic acid, dissociation (and the consequent inhibitory effect) is about ten times as great as for carbonic acid.

An H^+ concentration of 10^{-3} or 10^{-4} is just in the midst of H^+ concentration optima for various enzymes, as given by HÖBER (**6,** p. 721), varying from $10^{-1.5}$ for pepsin to $10^{-8.5}$ for esterase of the blood. No data are given for oxidase, but the work here reported on indicates that the optimum for them is much less than 10^{-3}. If the pyrogallol as used in the apparatus is just about the H^+ optimum, it is easily seen how the plant juice could make the H^+ concentration too high. Then when base is added, the strongest, most highly dissociated acids are neutralized first, and the H^+ concentration drops faster than represented by the degree of neutralization.

It may be possible to get some idea of the H^+ optimum for oxidase activity, and of the H^+ concentration of various mixtures in the apparatus by further work with indicators, but definite knowledge on these points must come finally from careful determinations by the gas-chain method.

Summary

1. Extract of apple tree bark affected with Illinois canker causes greater and more rapid oxidation of pyrogallol than does the extract of healthy bark.

2. Diseased bark extract is less acid than healthy bark extract, according to both indicator and titration figures, hence the

conclusion seems justified that oxidation is in approximately inverse ratio to the acidity of the extract in the range of concentrations here used.

3. This conclusion is borne out by the fact that addition of acid to the solution in the apparatus decreases oxidation and addition of alkali increases it.

4. Oxidases are very sensitive to small variations in acidity of the solution in the oxidase apparatus.

5. As a hypothesis in need of further proof the following is offered. The gradual slowing down of oxidation in the BUNZEL apparatus is brought about by accumulation of oxidation products, probably acetic and oxalic acids, and not by a using up of the oxidase through chemical combination between oxidase and oxidizable substance.

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